APPLICATION
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TITLE: **CHARACTERIZATION OF PHENYLALANINE AMMONIA-LYASE (PAL) GENE IN WOUNDED LETTUCE TISSUE**

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CHARACTERIZATION OF PHENYLALANINE AMMONIA-LYASE (PAL) GENE IN WOUNDED LETTUCE

Cross-reference to related applications

This application claims benefit of USSN 60/235,956 filed on September 26, 2000.

Background

The present invention is directed to nucleic acid and amino acid sequences, constructs comprising such sequences, and methods related thereto.

Introduction

Wounding is a common abiotic stress which induces altered protein synthesis in living tissue. Even slight mechanical injury induces the synthesis of enzymes responsible for a variety of wound responses in plants. For example, minimal processing of fresh fruit and vegetables involves many mechanical processes (e.g., abrading, cutting, peeling) which injure the tissue. Such wounding (e.g., cutting, cracking or breaking) induces alterations in many physiological processes which often make the processed item more perishable than the unprocessed fresh product and diminishes the shelf life of the final minimally processed product. Wounding also elicits several physiological responses associated with wound healing. Foremost among these reactions are changes in secondary metabolism and the concomitant increase in the propensity of tissue near the site of injury to brown.

Browning of fresh fruits and vegetables reduces quality and is often the factor limiting shelf life and marketability. This is especially true when these horticultural commodities are wounded by cutting, peeling, or abrading the surface during the preparation of minimally processed fresh fruits and vegetables. Enzymatic and non-enzymatic reactions with phenolic compounds produce brown pigments in plant tissue. Some tissues (e.g., artichokes) contain high levels of preformed phenolic compounds and rapidly brown in the air after wounding. Preventing browning in these tissues requires deactivation of the enzymes responsible for browning (e.g., polyphenoloxidase), exclusion of oxygen (e.g., oxygen levels below 1%), or application of chemical antioxidants (e.g., ascorbic acid). In other tissue, (e.g., lettuce) the quantity of phenolic compounds in uninjured tissue is low and browning follows the enhanced synthesis and accumulation of phenolic compounds.

The wounding of lettuce tissue induces the de novo synthesis of PAL, which initiates the reactions that lead to an increase in the level of phenolic compounds and browning. In

particular, wounding of lettuce produces a signal that migrates through the tissue and induces the synthesis of enzymes in the metabolic pathway responsible for increased production of phenolic compounds. New mRNA's can be measured and related to the synthesis and activation of a number of enzymes associated with phenylpropanoid metabolism. The first enzyme in the phenylpropanoid pathway is phenylalanine ammonia-lyase (PAL). Induced synthesis of this enzyme after wounding is rapidly followed by the accumulation of phenolic compounds like chlorogenic, isochlorogenic and dicaffeoyl tartaric acid, compounds that are associated with browning in lettuce. These phenolic compounds increase in wounded lettuce tissue, and are stored in the vacuole.

Wounding induces a rise in PAL activity that is time and temperature dependent, with the peak in wound-induced PAL activity being higher and occurring sooner at warmer temperatures. Methods used to control the increase in phenolic metabolism that leads to browning and loss of quality of minimally processed fresh produce include the use of reducing agents, enzyme inhibitors, acidulants, and complexing agents. Peiser, *et al.*, *Postharvest Biology and Technology* 14:171-177 (1998), demonstrated that control of PAL activity by inhibitors of PAL could control browning of cut lettuce. Some of these chemical treatments are very effective in controlling browning by interfering with specific metabolic pathways. For instance, o-benzylhydroxylamine, cysteine, and some phenylalanine analogues (e.g., 2-aminoindan-2 -phosphonic acid) have been reported to reduce the activity of enzymes associated with phenylpropanoid metabolism (e.g., PAL). However, concern about the use of chemicals and their toxic nature precludes their use on many minimally processed fresh fruits and vegetables. This concern with chemical residues is eliminated by the use of low oxygen, and or high carbon dioxide controlled and modified atmospheres (CA and MA), which are treatments currently employed in the commercial packaging of minimally processed lettuce. However, the use of CA requires special equipment for handling and storage, while the use of MA requires special equipment for packaging and expensive packaging material.

In U.S. Patent No. 6,113,958 the response of lettuce to wounding is minimized by the competitive metabolic induction of the heat shock response. Lettuce is subjected to heat-shock treatment at approximately 40° C to 60° C for approximately 360 seconds or less. At such temperatures there is a decrease in PAL activity from the redirection of the synthesis of proteins away from those related to wounding to those related to heat-shock. In this way heat-shock represses the synthesis of wound-induced enzymes of phenylpropanoid

metabolism and favors the synthesis of heat shock proteins. While this method is less expensive than CA or MA systems, it still requires investment in special equipment.

Therefore, there is a need for a method to produce plants having a modified response to wounding. There is also a continuing need for genetic engineering methods and tools that allow the manipulation of plant expression in response to stress events such as wounding. There is a particular need of non-constitutive promoters which can be used to tightly control the timing or tissue range and patterns of expression in response to damage from disease, or attack by a plant pest. Such a promoter would permit controlled expression of stress or disease-responsive proteins to the time and place of tissue damage, and/or the encounter with a pest or disease causing organism.

Summary of the Invention

The present invention is directed to the identification of phenylalanine ammonia-lyase encoding sequences, and in particular to phenylalanine ammonia-lyase (also referred to herein as PAL) sequences from the plant *Lactuca sativa*. The present invention also includes the production of anti-PAL antibodies produced using the isolated amino acid sequences of *L. sativa* PAL.

In one aspect, sequences are provided that are induced by wounding and which encode an enzyme capable of catalyzing the formation of trans-cinnamic acid by the deamination of L- phenylalanine.

In another aspect, sequences are provided encoding *Lactuca sativa* PAL enzyme and obtainable by polymerase chain reaction of paired degenerate primers GAYCCNYTNAAYTGGGG and CCYTGRAARTTNCCNCCRTG.

It is also an aspect of the present invention to provide recombinant DNA constructs that can be used for transcription or transcription and translation (expression) of PAL. In particular, constructs are provided which are capable of transcription or transcription and translation in host cells. Particularly preferred constructs are those capable of transcription or transcription and translation in plant cells.

In another aspect, the protein produced from the expression constructs are used as immunogens to produce either polyclonal or monoclonal antibodies specific for lettuce PAL proteins.

In another aspect of the present invention, methods are provided for modifying PAL levels in a host cell or progeny thereof. In particular, host cells are transformed or transfected

with a DNA construct which can be used for transcription or transcription and translation of PAL related sequences. The recombinant cells which contain PAL expressed by such a construct are also part of the present invention.

By the methods of this invention, the activity of PAL can be disrupted, and the browning response to wounding in vegetable crops can be reduced. Knowledge of the PAL sequence from a plant such as lettuce, which is susceptible to wound-induced browning, allows selected strategies for disrupting or down-regulating the PAL protein in plant cells. Such disruption may be provided by physical, chemical, or plant engineered methods.

In a preferred method, a construct may be employed having sequences associated with promoters which provide for expression of the PAL in response to wounding. Thus, in one aspect, the present invention relates to methods of using polynucleotide and polypeptide sequences to modify the browning associated with wound response in a plant host cell.

In another aspect, the invention provides promoters and methods for expressing proteins in a plant cell in response to wounding, and which have anti-fungal, anti-bacterial or anti-insecticidal activity.

The modified plants, seeds and crop obtained by plants having modified expression of PAL proteins are also considered part of the invention.

Brief Description Of The Drawings

Figure 1: Schematic presentation of the phenylpropanoid pathway wherein PAL is the first product in the pathway.

Figure 2: Comparison of the conserved regions of several known sequences to PAL, from sunflower (HA), *Arabadopsis* (AT), parsley (PC), carrot (DC), tobacco (NT), rice (OS) and wheat (TA), used to design primers for PCR (Genbank accession numbers in parenthesis).

Figure 3: Southern blot showing the products of PCR, with an approximately 1130 bp band detected after 20 hours.

Figure 4: The cloning strategy adopted to obtain the full-length cDNA.

Figure 5: SEQ ID NO:3, cDNA sequence to LsPAL1

Figure 6: SEQ ID NO:1, translated amino acid sequence of LsPAL1.

Figure 7: SEQ ID NO:4, cDNA sequence to LsPAL2.

Figure 8: SEQ ID NO:2, translated amino acid sequence of LsPAL2.

Figure 9: Sequence comparison of LsPAL1 to sunflower PAL demonstrating the close sequence homology.

Figure 10: Semi-quantitative LsPAL1 RNA expression demonstrating the temporal regulation of PAL1 in response to wounding. RNA levels peak at 12 hours post-wounding and decline to near baseline levels at 36 hours post-wounding.

Figure 11: Identification of wounding products from a 1cm piece of lettuce midrib, tested in three equal length segments 12 and 24 hours post-wounding. A) Cinnamic acid concentration at 12 and 24 hours compared to control lettuce segments. Cinnamic acid is the second product produced in the phenylpropanoid pathway as depicted in figure 1. B) LsPAL1 expression 12 hours post-wounding compared to control lettuce segments.

Figure 12: Distribution of LsPAL1 RNA in epidermal, vascular and cortex lettuce tissue in response to wounding.

Detailed Description of the Invention

In accordance with the subject invention, nucleic acid and protein sequences obtainable from a plant source are provided which are capable of catalyzing the formation of trans-cinnamic acid by the deamination of L- phenylalanine. Such proteins are referred to herein as phenylalanine ammonia-lyase proteins, or PAL.

Numerous reports in the literature detail how the activity of the phenylpropanoid pathway in plants is increased following abiotic and biotic stresses. Increased activity of this pathway results in the synthesis and accumulation of phenolic compounds that contribute to would healing, plant defense and tissue browning. The first committed enzyme in this pathway is phenylalanine ammonia-lyase (PAL), which also controls the rate at which phenolic compounds are produced by this and subsequent pathways. Possession of the gene allows its manipulation by genetic engineering techniques to enhance or suppress its action. Tissue can now be produced with enhanced disease resistance, or demonstrating suppressed browning potential following wounding.

The peptide sequences provided are useful for obtaining polynucleotide sequences which encode PAL, and sequences associated with the expression of PAL in response to wounding. The obtained nucleic acid sequences find use in the preparation of constructs to direct their expression in a host cell. The sequences also provide means for adopting strategies to use physical or chemical methods to inactivate or disrupt the PAL activity, or expression of the PAL protein.

Furthermore, the nucleic acid sequences find use in the preparation of plant expression constructs to modify other features of the response of a plant cell to wounding, damage or other injury from disease-causing organisms or the actions of a plant pest

Though it is known that PAL activity is highest at 24 hours post wounding, LsPAL1 mRNA accumulation peaks at 12 hours after wounding. Additionally, signals induced by wounding trigger LsPAL1 mRNA expression in tissues not directly damaged. LsPAL1 mRNA is mainly expressed in tissue close to the epidermis and vascular tissue. Genomic sequences associated with expression of LsPAL1 can be used to direct expression in response to the wounding stress. Such a promoter is well-suited to expression of peptides used to counter or control the progression of pathogens and pests at the site of infestation.

The invention also includes polypeptides of the formula:

$$X\text{-}(R_1)_n\text{-}(R_2)\text{-}(R_3)_n\text{-}Y$$

wherein, at the amino terminus, X is hydrogen, and at the carboxyl terminus, Y is hydrogen or a metal, R_1 and R_3 are any amino acid residue, n is an integer between 1 and 1000, and R_2 is an amino acid sequence of the invention, particularly an amino acid sequence selected from the group set forth in Figure 6. In the formula, R_2 is oriented so that its amino terminal residue is at the left, bound to R_1, and its carboxy terminal residue is at the right, bound to R_3. Any stretch of amino acid residues denoted by either R group, where R is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer.

Polypeptides of the present invention include isolated polypeptides encoded by a polynucleotide comprising a sequence selected from the group of a sequence contained in Figure 5, and sequences encoding the amino acid sequence of Figure 6 (SEQ ID NOs: 1 and 2, respectively). The open reading frame begins at the ATG at base 119, and continues to the stop at 2254.

Polypeptides of the present invention have been shown to be associated with phenylalanine ammonia-lyase activity and are of interest because PAL is involved in the production of secondary-metabolites in the wound response of plants.

The polypeptides of the present invention can be mature protein or can be part of a fusion protein.

Fragments and variants of the polypeptides are also considered to be a part of the invention. A fragment is a variant polypeptide which has an amino acid sequence that is entirely the same as part but not all of the amino acid sequence of the previously described polypeptides. The fragments can be "free-standing" or comprised within a larger polypeptide of which the fragment forms a part or a region, most preferably as a single continuous region

Preferred fragments are biologically active fragments which are those fragments that mediate activities of the polypeptides of the invention, including those with similar activity or improved activity or with a decreased activity. Also included are those fragments that antigenic or immunogenic in an animal, particularly a human.

Variants of the polypeptide also include polypeptides that vary from the sequences set forth in the Sequence Listing by conservative amino acid substitutions, substitution of a residue by another with like characteristics. In general, such substitutions are among Ala, Val, Leu and Ile; between Ser and Thr; between Asp and Glu; between Asn and Gln; between Lys and Arg; or between Phe and Tyr. Particularly preferred are variants in which 5 to 10; 1 to 5; 1 to 3 or one amino acid(s) are substituted, deleted, or added, in any combination.

Variants that are fragments of the polypeptides of the invention can be used to produce the corresponding full length polypeptide by peptide synthesis. Therefore, these variants can be used as intermediates for producing the full-length polypeptides of the invention.

A nucleic acid sequence of a PAL of this invention may be a DNA or RNA sequence, derived from genomic DNA, cDNA, mRNA, or may be synthesized in whole or in part. The gene sequences may be cloned, for example, by isolating genomic DNA from an appropriate source, and amplifying and cloning the sequence of interest using a polymerase chain reaction (PCR). Alternatively, the gene sequences may be synthesized, either completely or in part, especially where it is desirable to provide plant-preferred sequences. Thus, all or a portion of the desired structural gene (that portion of the gene which encodes the PAL protein) may be synthesized using codons preferred by a selected host. Host-preferred codons may be determined, for example, from the codons used most frequently in the proteins expressed in a desired host species.

Thus also considered in the present invention are isolated PAL polynucleotides obtained from the polypeptide sequences of the present invention. Such polynucleotide sequences include isolated polynucleotides that encode the polypeptides of the invention having a deduced amino acid sequence selected from the group of sequences set forth in the Sequence Listing and to other polynucleotide sequences closely related to such sequences and variants thereof.

The invention provides a polynucleotide sequence identical over its entire length to each coding sequence as set forth in the Sequence Listing. The invention also provides the coding sequence for the mature polypeptide or a fragment thereof, as well as the coding sequence for the mature polypeptide or a fragment thereof in a reading frame with other

coding sequences, such as those encoding a leader or secretory sequence, a pre-, pro-, or prepro- protein sequence. The polynucleotide can also include non-coding sequences, including for example, but not limited to, non-coding 5' and 3' sequences, such as the transcribed, untranslated sequences, termination signals, ribosome binding sites, sequences that stabilize mRNA, introns, polyadenylation signals, and additional coding sequence that encodes additional amino acids. For example, a marker sequence can be included to facilitate the purification of the fused polypeptide. Polynucleotides of the present invention also include polynucleotides comprising a structural gene and the naturally associated sequences that control gene expression.

The invention also includes polynucleotides of the formula:

$$X\text{-}(R_1)_n\text{-}(R_2)\text{-}(R_3)_n\text{-}Y$$

wherein, at the 5' end, X is hydrogen, and at the 3' end, Y is hydrogen or a metal, R_1 and R_3 are any nucleic acid residue, n is an integer between 1 and 3000, preferably between 1 and 1000 and R_2 is a nucleic acid sequence of the invention, particularly a nucleic acid sequence selected from the sequence of SEQ ID NO: 1 and nucleic acid sequences encoding the peptide of SEQ ID NO: 2. In the formula, R_2 is oriented so that its 5' end residue is at the left, bound to R_1, and its 3' end residue is at the right, bound to R_3. Any stretch of nucleic acid residues denoted by either R group, where R is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer.

The invention also relates to variants of the polynucleotides described herein that encode for variants of the polypeptides of the invention. Variants that are fragments of the polynucleotides of the invention can be used to synthesize full-length polynucleotides of the invention. Preferred embodiments are polynucleotides encoding polypeptide variants wherein 5 to 10, 1 to 5, 1 to 3, 2, 1 or no amino acid residues of a polypeptide sequence of the invention are substituted, added or deleted, in any combination. Particularly preferred are substitutions, additions, and deletions that are silent such that they do not alter the properties or activities of the polynucleotide or polypeptide.

The invention also provides a polynucleotide consisting essentially of a polynucleotide sequence obtainable by screening an appropriate library containing the complete gene for a polynucleotide sequence set for in the Sequence Listing under stringent hybridization conditions with a probe having the sequence of said polynucleotide sequence or a fragment thereof, and isolating said polynucleotide sequence. Fragments useful for obtaining such a polynucleotide include, for example, probes and primers as described herein.

As discussed herein regarding polynucleotide assays of the invention, for example, polynucleotides of the invention can be used as a hybridization probe for RNA, cDNA, or genomic DNA to isolate full length cDNAs or genomic clones encoding a polypeptide and to isolate cDNA or genomic clones of other genes that have a high sequence similarity to a polynucleotide set forth in the Sequence Listing. Such probes will generally comprise at least 15 bases. Preferably such probes will have at least 30 bases and can have at least 50 bases. Particularly preferred probes will have between 30 bases and 50 bases, inclusive.

As discussed above, nucleic acid sequence encoding a PAL of this invention may include genomic, cDNA or mRNA sequence. By "encoding" is meant that the sequence corresponds to a particular amino acid sequence either in a sense or anti-sense orientation. By "extrachromosomal" is meant that the sequence is outside of the plant genome of which it is naturally associated. By "recombinant" is meant that the sequence contains a genetically engineered modification through manipulation via mutagenesis, restriction enzymes, and the like.

By "genomic sequences" of the invention, it is meant to include those flanking sequences associated with the timing and manner of expression of PAL, particularly those sequences which regulate the expression of LsPAL1 in response to wounding, or in a "wound-induced" fashion. Such sequences will find many applications, including the expression of pest and pathogen resistance proteins in plant tissue. In this manner plants may be produced with increased resistance to bacterial and fungal infestations, and also having resistance to infestation by insect pests.

The coding region of each gene that comprises or is comprised by a polynucleotide sequence set forth in the Sequence Listing may be isolated by screening using a DNA sequence provided in the Sequence Listing to synthesize an oligonucleotide probe. A labeled oligonucleotide having a sequence complementary to that of a gene of the invention is then used to screen a library of cDNA, genomic DNA or mRNA to identify members of the library that hybridize to the probe. For example, synthetic oligonucleotides are prepared which correspond to the N-terminal sequence of the polypeptide. The partial sequences so prepared can then be used as probes to obtain PAL clones from a gene library prepared from a cell source of interest. Alternatively, where oligonucleotides of low degeneracy can be prepared from particular peptides, such probes may be used directly to screen gene libraries for gene sequences. In particular, screening of cDNA libraries in phage vectors is useful in such methods due to lower levels of background hybridization.

The polynucleotides and polypeptides of the invention can be used, for example, in the transformation of various host cells, as further discussed herein

The invention also provides polynucleotides that encode a polypeptide that is a mature protein plus additional amino or carboxyl-terminal amino acids, or amino acids within the mature polypeptide (for example, when the mature form of the protein has more than one polypeptide chain). Such sequences can, for example, play a role in the processing of a protein from a precursor to a mature form, allow protein transport, shorten or lengthen protein half-life, or facilitate manipulation of the protein in assays or production. It is contemplated that cellular enzymes can be used to remove any additional amino acids from the mature protein.

A precursor protein, having the mature form of the polypeptide fused to one or more prosequences may be an inactive form of the polypeptide. The inactive precursors generally are activated when the prosequences are removed. Some or all of the prosequences may be removed prior to activation. Such precursor protein are generally called proproteins.

The polynucleotide and polypeptide sequences can also be used to identify additional sequences which are homologous to the sequences of the present invention. The most preferable and convenient method is to store the sequence in a computer readable medium, for example, floppy disk, CD ROM, hard disk drives, external disk drives and DVD, and then to use the stored sequence to search a sequence database with well known searching tools. Examples of public databases include the DNA Database of Japan (DDBJ)(http://www.ddbj.nig.ac.jp/); Genbank (http://www.ncbi.nlm.nih.gov/web/Genbank/Index.html); and the European Molecular Biology Laboratory Nucleic Acid Sequence Database (EMBL) (http://www.ebi.ac.uk/ebi_docs/embl_db.html). A number of different search algorithms are available to the skilled artisan, one example of which are the suite of programs referred to as BLAST programs. There are five implementations of BLAST, three designed for nucleotide sequences queries (BLASTN, BLASTX, and TBLASTX) and two designed for protein sequence queries (BLASTP and TBLASTN) (Coulson, *Trends in Biotechnology*, 12:76-80 (1994); Birren, *et al.*, *Genome Analysis*, 1:543-559 (1997)). Additional programs are available in the art for the analysis of identified sequences, such as sequence alignment programs, programs for the identification of more distantly related sequences, and the like, and are well known to the skilled artisan.

Of interest in the present invention, is the use of the nucleotide sequences, or polynucleotides, in recombinant DNA constructs to direct the transcription or transcription and translation (expression) of the PAL sequences of the present invention in a host cell.

As used herein, "recombinant" includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid sequence or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all as a result of deliberate human intervention.

Of particular interest is the use of the nucleotide sequences, or polynucleotides, in recombinant DNA constructs to direct the transcription or transcription and translation (expression) of the phenylalanine ammonia-lyase sequences of the present invention in a host cell. The expression constructs generally comprise a promoter functional in a host cell operably linked to a nucleic acid sequence encoding a phenylalanine ammonia-lyase of the present invention and a transcriptional termination region functional in a host cell.

By "host cell" is meant a cell which contains a vector and supports the replication, and/or transcription or transcription and translation (expression) of the expression construct. Host cells for use in the present invention can be prokaryotic cells, such as *E. coli*, or eukaryotic cells such as yeast, plant, insect, amphibian, or mammalian cells. Preferably, host cells are monocotyledenous or dicotyledenous plant cells.

Of particular interest in the present invention is the use of the polynucleotides of the present invention for the preparation of constructs to direct the transcription or transcription and translation of the nucleotide sequences encoding phenylalanine ammonia-lyase in a host plant cell. Plant expression constructs generally comprise a promoter functional in a plant host cell operably linked to a nucleic acid sequence of the present and a transcriptional termination region functional in a host plant cell.

Those skilled in the art will recognize that there are a number of promoters which are functional in plant cells, and have been described in the literature. Chloroplast and plastid specific promoters, chloroplast or plastid functional promoters, and chloroplast or plastid operable promoters are also envisioned.

One set of promoters is constitutive promoters such as the CaMV35S or FMV35S promoters that yield high levels of expression in most plant organs. Enhanced or duplicated versions of the CaMV35S and FMV35S promoters are useful in the practice of this invention (Odell, *et al.* (1985) *Nature* 313:810-812; Rogers, U.S. Patent Number 5,378,619). In

addition, it may also be preferred to bring about expression of the protein of interest in specific tissues of the plant, such as leaf, stem, root, tuber, seed, fruit, etc., and the promoter chosen should have the desired tissue and developmental specificity.

Of particular interest is the expression of the nucleic acid sequences of the present invention from transcription initiation regions which are preferentially expressed in wounded tissue. In this manner, the promoter region naturally associated with the LsPAL1 sequence may be used.

It may be advantageous to direct the localization of proteins conferring phenylalanine ammonia-lyase to a particular subcellular compartment, for example, to the mitochondrion, endoplasmic reticulum, vacuoles, chloroplast or other plastidic compartment. For example, where the genes of interest of the present invention will be targeted to plastids, such as chloroplasts, for expression, the constructs will also employ the use of sequences to direct the gene to the plastid. Such sequences are referred to herein as chloroplast transit peptides (CTP) or plastid transit peptides (PTP). In this manner, where the gene of interest is not directly inserted into the plastid, the expression construct will additionally contain a gene encoding a transit peptide to direct the gene of interest to the plastid. The chloroplast transit peptides may be derived from the gene of interest, or may be derived from a heterologous sequence having a CTP. Such transit peptides are known in the art. See, for example, Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep.* 9:104-126; Clark *et al.* (1989) *J. Biol. Chem.* 264:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res Commun.* 196:1414-1421; and, Shah *et al.* (1986) *Science* 233:478-481. Additional transit peptides for the translocation of the protein to the endoplasmic reticulum (ER) (Chrispeels, K., (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol.* 42:21-53), nuclear localization signals (Raikhel, N. (1992) *Plant Phys.* 100:1627-1632), or vacuole may also find use in the constructs of the present invention.

Depending upon the intended use, the constructs may contain the nucleic acid sequence which encodes the entire phenylalanine ammonia-lyase protein, or a portion thereof. For example, where antisense inhibition of a given PAL protein is desired, the entire phenylalanine ammonia-lyase sequence is not required. Furthermore, where PAL sequences used in constructs are intended for use as probes, it may be advantageous to prepare constructs containing only a particular portion of a phenylalanine ammonia-lyase encoding sequence, for example a sequence which is discovered to encode a highly conserved PAL region.

The skilled artisan will recognize that there are various methods for the inhibition of expression of endogenous sequences in a host cell. Such methods include, but are not limited to antisense suppression (Smith, *et al.* (1988) *Nature* 334:724-726), co-suppression (Napoli, *et al.* (1989) *Plant Cell* 2:279-289), ribozymes (PCT Publication WO 97/10328), and combinations of sense and antisense Waterhouse, *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95:13959-13964. Methods for the suppression of endogenous sequences in a host cell typically employ the transcription or transcription and translation of at least a portion of the sequence to be suppressed. Such sequences may be homologous to coding as well as non-coding regions of the endogenous sequence.

Regulatory transcript termination regions may be provided in plant expression constructs of this invention as well. Transcript termination regions may be provided by the DNA sequence encoding the phenylalanine ammonia-lyase or a convenient transcription termination region derived from a different gene source, for example, the transcript termination region which is naturally associated with the transcript initiation region. The skilled artisan will recognize that any convenient transcript termination region which is capable of terminating transcription in a plant cell may be employed in the constructs of the present invention.

Alternatively, constructs may be prepared to direct the expression of the phenylalanine ammonia-lyase sequences directly from the host plant cell plastid. Such constructs and methods are known in the art and are generally described, for example, in Svab, *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:8526-8530 and Svab and Maliga (1993) *Proc. Natl. Acad. Sci. USA* 90:913-917 and in U.S. Patent Number 5,693,507.

A plant cell, tissue, organ, or plant into which the recombinant DNA constructs containing the expression constructs have been introduced is considered transformed, transfected, or transgenic. A transgenic or transformed cell or plant also includes progeny of the cell or plant and progeny produced from a breeding program employing such a transgenic plant as a parent in a cross and exhibiting an altered phenotype resulting from the presence of a PAL nucleic acid sequence. Hence, a plant of the invention will include any plant which has a cell containing a construct with introduced nucleic acid sequences, regardless of whether the sequence was introduced into the cell directly through transformation means or introduced by generational transfer from a progenitor cell which originally received the construct by direct transformation

The term "introduced" in the context of inserting a nucleic acid sequence into a cell, means "transfection", or "transformation" or "transduction" and includes reference to the

incorporation of a nucleic acid sequence into a eukaryotic or prokaryotic cell where the nucleic acid sequence may be incorporated into the genome of the cell (for example, chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (for example, transfected mRNA).

Plant expression or transcription constructs having a phenylalanine ammonia-lyase as the DNA sequence of interest for increased or decreased expression thereof may be employed with a wide variety of plant life, particularly, plant life involved in the production of improved produce crops. Plants of interest in the present invention include monocotyledenous and dicotyledenous plants. Most especially preferred are plants from which produce crops are obtained. Plants of interest include, but are not limited to, lettuce, celery, spinach and green bean. Depending on the method for introducing the recombinant constructs into the host cell, other DNA sequences may be required.

As used herein, the term "plant" includes reference to whole plants, plant organs (for example, leaves, stems, roots, etc.), seeds, and plant cells and progeny of same. Plant cell, as used herein includes, without limitation, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves roots shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants which can be used in the methods of the present invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledenous and dicotyledenous plants.

As used herein, "transgenic plant" includes reference to a plant which comprises within its genome a heterologous polynucleotide. Generally, the heterologous polynucleotide is stably integrated within the genome such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant expression cassette. "Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant, the genotype of which has been altered by the presence of heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

Thus a plant having within its cells a heterologous polynucleotide is referred to herein as a transgenic plant. The heterologous polynucleotide can be either stably integrated into the

genome, or can be extra-chromosomal. Preferably, the polynucleotide of the present invention is stably integrated into the genome such that the polynucleotide is passed on to successive generations. The polynucleotide is integrated into the genome alone or as part of a recombinant expression cassette. "Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant, the genotype of which has been altered by the presence of heterologous nucleic acids including those transgenics initially so altered as well as those created by sexual crosses or asexual reproduction of the initial transgenics.

As used herein, "heterologous" in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous structural gene is from a species different from that from which the structural gene was derived, or, if from the same species, one or both are substantially modified from their original form. A heterologous protein may originate from a foreign species, or, if from the same species, is substantially modified from its original form by deliberate human intervention.

As used herein, a "recombinant expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements which permit transcription of a particular nucleic acid in a target cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid sequence to be transcribed and a promoter.

Of interest in the present invention, is the use of phenylalanine ammonia-lyase (PAL) constructs in plants in order to alter or modulate the plants response to wounding . Hence, in another aspect, a method is provided for controlling a plants response to wounding. In a preferred such method, plants are provided which demonstrate a greatly diminished wound-induced browning. Plants such as lettuce, celery, green bean and spinach are particularly preferred for use with this method. Crop harvested from such plants is also considered herein.

It is contemplated that the gene sequences may be synthesized, either completely or in part, especially where it is desirable to provide plant-preferred sequences. Thus, all or a portion of the desired structural gene (that portion of the gene which encodes the PAL protein) may be synthesized using codons preferred by a selected host. Host-preferred

codons may be determined, for example, from the codons used most frequently in the proteins expressed in a desired host species.

One skilled in the art will readily recognize that antibody preparations, nucleic acid probes (DNA and RNA) and the like may be prepared and used to screen and recover "homologous" or "related" PAL from a variety of plant sources. Homologous sequences are found when there is an identity of sequence, which may be determined upon comparison of sequence information, nucleic acid or amino acid, or through hybridization reactions between a known PAL and a candidate source. Conservative changes, such as Glu/Asp, Val/Ile, Ser/Thr, Arg/Lys and Gln/Asn may also be considered in determining sequence homology. Amino acid sequences are considered homologous by as little as 25% sequence identity between the two complete mature proteins. (*See* generally, Doolittle, R.F., *OF URFS and ORFS* (University Science Books, CA, 1986.)

The nucleic acid sequences associated with phenylalanine ammonia-lyase proteins will find many uses. For example, recombinant constructs can be prepared which can be used as probes, or which will provide for expression of the PAL protein in host cells to produce a ready source of the enzyme and/or to modify the wound response of the plant. Other useful applications may be found when the host cell is a plant host cell, either *in vitro* or *in vivo*. For example, by expressing a PAL protein in a host plant cell, altered responses to wounding may be produced in a given plant tissue. In a like manner, for some applications it may be desired to decrease the amount of PAL endogenously expressed in a plant cell by various gene suppression technologies discussed *supra*.

Once the desired PAL nucleic acid or promoter sequence is obtained, it may be manipulated in a variety of ways. Where the sequence involves non-coding flanking regions, the flanking regions may be subjected to resection, mutagenesis, etc. Thus, transitions, transversions, deletions, and insertions may be performed on the naturally occurring sequence. In addition, all or part of the sequence may be synthesized. In the structural gene, one or more codons may be modified to provide for a modified amino acid sequence, or one or more codon mutations may be introduced to provide for a convenient restriction site or other purpose involved with construction or expression. The structural gene may be further modified by employing synthetic adapters, linkers to introduce one or more convenient restriction sites, or the like.

The nucleic acid or amino acid sequences encoding a PAL of this invention may be combined with other non-native, or "heterologous", sequences in a variety of ways. By "heterologous" sequences is meant any sequence which is not naturally found joined to the

PAL, including, for example, combinations of nucleic acid sequences from the same plant which are not naturally found joined together.

The DNA sequence encoding a phenylalanine ammonia-lyase of this invention may be employed in conjunction with all or part of the gene sequences normally associated with the PAL. In its component parts, a DNA sequence encoding PAL is combined in a DNA construct having, in the 5' to 3' direction of transcription, a transcription initiation control region capable of promoting transcription and translation in a host cell, the DNA sequence encoding plant PAL and a transcription and translation termination region.

Potential host cells include both prokaryotic cells, such as *E.coli* and eukaryotic cells such as yeast, insect, amphibian, or mammalian cells. A host cell may be unicellular or found in a multicellular differentiated or undifferentiated organism depending upon the intended use. Preferably, host cells of the present invention include plant cells, both monocotyledenous and dicotyledenous. Cells of this invention may be distinguished by having a PAL foreign to the wild-type cell present therein, for example, by having a recombinant nucleic acid construct encoding a PAL therein.

The methods used for the transformation of the host plant cell are not critical to the present invention. The transformation of the plant is preferably permanent, *i.e.* by integration of the introduced expression constructs into the host plant genome, so that the introduced constructs are passed onto successive plant generations. The skilled artisan will recognize that a wide variety of transformation techniques exist in the art, and new techniques are continually becoming available. Any technique that is suitable for the target host plant can be employed within the scope of the present invention. For example, the constructs can be introduced in a variety of forms including, but not limited to as a strand of DNA, in a plasmid, or in an artificial chromosome. The introduction of the constructs into the target plant cells can be accomplished by a variety of techniques, including, but not limited to calcium-phosphate-DNA co-precipitation, electroporation, microinjection, *Agrobacterium* infection, liposomes or microprojectile transformation. The skilled artisan can refer to the literature for details and select suitable techniques for use in the methods of the present invention.

Normally, included with the DNA construct will be a structural gene having the necessary regulatory regions for expression in a host and providing for selection of transformant cells. The gene may provide for resistance to a cytotoxic agent, *e.g.* antibiotic, heavy metal, toxin, etc., complementation providing prototrophy to an auxotrophic host, viral immunity or the like. Depending upon the number of different host species the expression

construct or components thereof are introduced, one or more markers may be employed, where different conditions for selection are used for the different hosts

Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the *Agrobacterium* host for homologous recombination with T-DNA or the Ti- or Ri-plasmid present in the *Agrobacterium* host. The Ti- or Ri-plasmid containing the T-DNA for recombination may be armed (capable of causing gall formation) or disarmed (incapable of causing gall formation), the latter being permissible, so long as the *vir* genes are present in the transformed *Agrobacterium* host. The armed plasmid can give a mixture of normal plant cells and gall.

In some instances where *Agrobacterium* is used as the vehicle for transforming host plant cells, the expression or transcription construct bordered by the T-DNA border region(s) will be inserted into a broad host range vector capable of replication in *E. coli* and *Agrobacterium*, there being broad host range vectors described in the literature. Commonly used is pRK2 or derivatives thereof. See, for example, Ditta, *et al.*, (*Proc. Nat. Acad. Sci., U.S.A.* (1980) 77:7347-7351) and EPA 0 120 515, which are incorporated herein by reference. Alternatively, one may insert the sequences to be expressed in plant cells into a vector containing separate replication sequences, one of which stabilizes the vector in *E. coli*, and the other in *Agrobacterium*. See, for example, McBride and Summerfelt (*Plant Mol. Biol.* (1990) 14:269-276), wherein the pRiHRI (Jouanin, *et al.*, *Mol. Gen. Genet.* (1985) 201:370-374) origin of replication is utilized and provides for added stability of the plant expression vectors in host *Agrobacterium* cells.

Included with the expression construct and the T-DNA will be one or more markers, which allow for selection of transformed Agrobacterium and transformed plant cells. A number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, kanamycin, the aminoglycoside G418, hygromycin, or the like. The particular marker employed is not essential to this invention, one or another marker being preferred depending on the particular host and the manner of construction.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an appropriate selective medium. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be grown to seed and the seed used to establish repetitive generations and for production of improved produce crop

There are several possible ways to obtain the plant cells of this invention which contain multiple expression constructs. Any means for producing a plant comprising a construct having a nucleic acid sequence of the present invention, and at least one other construct having another DNA sequence encoding an enzyme are encompassed by the present invention. For example, the expression construct can be used to transform a plant at the same time as the second construct either by inclusion of both expression constructs in a single transformation vector or by using separate vectors, each of which express desired genes. The second construct can be introduced into a plant which has already been transformed with the first expression construct, or alternatively, transformed plants, one having the first construct and one having the second construct, can be crossed to bring the constructs together in the same plant.

The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

Examples

Example 1: Identification of Lettuce PAL1 and PAL2 DNA sequences

Phenylalanine ammonia-lyase is highly conserved in plants, figure 2, therefore degenerate primers were developed to identify PAL genes in lettuce. As per Figure 2, degenerate primers were designed for polymerase chain reaction (PCR) based on peptide sequences which were similar among sunflower, *Arabidopsis*, parsley, carrot, tobacco, wheat and rice sequences. The peptide sequences chosen for PCR include a region near the 5' end of the PAL encoding sequence, peptide fragment DPLNW, and a sequence approximately one-third from the 3' end, encoding the peptide fragment HGGNFQG.

The degenerate primers produced for PCR from these peptide fragments were GAYCCNYTNAAYTGGGG (5') and CCYTGRAARTTNCCNCCRTG (3'). These primers were used to PCR amplify a portion of the open reading frame (ORF) from a *Lactuca sativa* cDNA library. The above primer pairs yielded PCR product which was in the expected range of 1.1 kb (Figure 3). The PCR products were then cloned into a vector which is amplified by expression of the cloned genes in bacteria. Bacterial colonies were selected and checked for the presence of vector insertions. DNA was then purified from the bacterial colonies.

There are several methods available and well know to the skilled artisan to obtain full-length cDNAs, or extend short cDNAs, for example those based on the method of Rapid Amplification of cDNA Ends (RACE) (see, for example, Frohman *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:8998-9002). Recent modifications of the technique, exemplified by the Marathon™ technology (Clonetech Laboratories, Inc.) for example, have significantly simplified obtaining full-length cDNA sequences.

The resulting individual clones had a single-stranded sequence that was extended using a 3' RACE reaction. The reaction (depicted in Figure 4) yielded a 2.4 kb product which was again cloned into a vector and double-stranded sequence obtained and sequenced.

The sequences (Figure 5 and 7) reveals the presence of a start and stop codon indicating the entire open reading frame of the gene is cloned. Figure 5 and 7 depicts the DNA sequence to this clone, and Figure 6 and 8 the translated protein sequence of the gene product. The two full-length clones that were identified were designated LsPAL1 and LsPAL2.

Results of a database search using BLAST indicate LsPAL1 is highly homologous to sunflower PAL sequence (Figure 9), except for a missing terminal amino acid series from sunflower which is found in LsPAL1.

Example 2: Expression of the fusion protein.

The methodology used for expression and purification of the fusion protein, MBP-PAL1, in *E.coli* was made following the procedures shown by Nonogaki et al. (2000) with differences explained as follow. Two primers were designed which complement the protein-encoded sequence of LsPAL1. The forward primer (5'-CGGAATTCATGGAGAACGGTAAT-3') included an EcorI site, while the reverse primer (5'-CGTCTAGACTAACATATTGGAAG-3') incorporated an XbaI site. The PAL open reading frame was cloned into the EcorI and XbaI site in pMALc vector (New England Labs, MN). The transformed bacteria were incubated overnight at 37 °C. An aliquot of the overnight culture was used to inoculate an incubation broth for 4 h at 37 °C. The cells were harvested by centrifugation and resuspended in sonication buffer (Nonogaki et al., 2000). After freezing overnight, the cells were thawed and sonicated for 5-10 min to release a higher amount of soluble fusion protein. The soluble protein was purified as Nonogaki et al. (2000)

and separated by electrophoresis in a 10% acrylamide gel. The bands were stained with Coomassie brilliant blue (Fisher, PA) for approximately 1 h, and de-stained to visualize the major bands. A pre-stained broad range protein standard (Bio-Rad, CA) was used to estimate the molecular weights

Example 3: Fusion protein demonstrates PAL activity

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activity was measured as previously described by Ke and Saltveit (1986).

Example 4: Measurement of Cinnamic acid of the phenylpropanoid pathway in lettuce tissue

Heads of Romaine lettuce (*Lactuca sativa* L., var. Longifolia) were obtained from commercial sources, transported to the Univ. of Calif., Davis, Mann Laboratory and held at 0.5 °C until used. Complete leaves or 1-cm cross-sections of the trimmed mid-ribs were used in the experiments. The leaves used in all experiments were chosen from the middle of the head, eliminating the outer leaves and the center. Pieces were cut from the achlorophyllous mid-rib with a stainless steel razor blade and store at 10 °C for variable times to evaluated the kinetics of induced PAL activity, phenolic determinations, gene expression and protein accumulation.

The concentration of phenolic compounds was measured as described by Ke and Saltveit (1988). Briefly, 10 g of tissue, control and wounded, was stored for 48 h at 10 °C, and then ground in 20 ml of methanol HPLC grade with the Ultra-Turrax tissue homogenizer. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15000 x g for 20 min. As described by Loiza-Velarde et al. (1997), the absorbance of an aliquot of the supernatant was read at 320 nm (potential browning) and 437 nm (soluble o-quinones) using an UV-VIS spectrophotometer (Shimadzu UV-160A). The remnants of the extraction were placed in a Multiwell tissue culture plate (Falcon 3047, Becton Dickinson) and color evaluation were done as described by Loaiza-Velarde and Saltveit (2001). The L*, a* and b* values were recorded using a colorimeter (CR-200, Minolta).

Results are shown in figure 11.

Example 5: Identification of PAL expression in wounded lettuce tissue

Total RNA was extracted from wounded lettuce at 0, 2, 8, 12, 24 and 36 hours after wounding to evaluate the temporal expression of LsPAL1 in wounded tissue. As seen in Figure 10, at 10 °C, PAL mRNA accumulates to a peak at 12 h post-wounding, and then declines steadily thereafter

Total RNA was also extracted from wounded lettuce from segments taken from a 1cm cut piece of lettuce midrib. As seen in Figure 11, the center "uninjured" third of the 1 cm segment accumulates PAL mRNA at 24 hours, demonstrating that the wound signal is effective at some distance from the site of wounding.

Total RNA was extracted from achlorophyllous mid-rib lettuce tissue of mature leaves. A phenol extraction method was performed as described by Sambrook et al. (1989). The total RNA electrophoreses was performed in 1.3% (w/v) agarose gels with 7% (v/v) formaldehyde. The gels were transferred overnight to Hybond N+ membrane (Amersham Pharmacia, NJ) and UV cross-linked. Riboprobes were generated using DIG labeled dNTP (Boehringer Mannheim, Indianapolis). The probe corresponded to the first 382 bp of the LsPAL1 gene (GeneBank accession number AF299330) cloned in pBIIKS (Stratagene, CA). The membranes were pre-hybridized for 30 min in a buffer composed by 5x SSC, 50% (v/v) formamide, 4%(w/v) blocking reagent (Boehringer Mannheim, Indianapolis), 0.2% (w/v) SDS, 0.1% (w/v) N-lauroylsarcosine. The hybridization and washing of the membranes and the chemiluminescence detection of the signal was done as described by Nonogaki et al. (2000). The quantification of the relative expression of signal was performed by densitometer scanning (IS-1000 Digital Imaging System, Alpha Innotech Corporation) integration of the signal of the northern blot normalized by the integration of the ethidium bromide stained ribosomal RNA of the same sample.

Evaluations of the localization of LsPAL1 mRNA demonstrates that it is differentially expressed. Epidermal and vascular tissues near the cut surface show the greatest accumulation of PAL mRNA after wounding (Figure 12). Cortex tissue near the cut surface and epidermal and vascular tissue farther away from the cut surface showed much less accumulation PAL mRNA 24 hours after wounding.

Example 6: Identification of PAL protein expression following wounding

Soluble proteins from lettuce mid rib were extracted with the same buffer and similar methodology than for PAL activity assay. The supernatant was mixed 1:2 (v/v) with cold acetone (-20 °C) and kept at that temperature for 1 h. The pellet was air dried and re-

suspended in 50mM PBS. The suspension was centrifuged in a bench top centrifuge at maximum speed for 5 min to remove insoluble fraction. A sample of the supernatant was assayed with the Bradford reagent (Bio-Rad, CA) using bovine gamma globulin (Bio-Rad, CA) as standard. The protein concentration of the samples was adjusted by diluting them with PBS

The separation of the proteins was performed by SDS-PAGE in a 10% (w/v) acrylamide gels as described by Laemmli (1970). A Mini-Protean II (Bio-Rad, CA) electrophoresis system was used. The gels were loaded with equal amount (10 µg) of soluble total protein per well, which were previously kept for 5 min at 90-95°C. A pre-stained broad range protein standard (Bio-Rad, CA) was used to estimate the molecular weights. After electrophoresis, the proteins were blotted, hybridized, washed and signal detected as Nonogaki et al. (2000). A brief descriptions as followed. The proteins were electrobloted on polyvinylidine difluoride (PVDF) membranes (Immobilon-P, Millipore, MA) using a semidry blotter (Bio Rad, CA). The membranes were blocked for 1 h at 25 °C or overnight at 5 °C with 5% (w/v) skim milk in 50 mM phosphate buffered saline (PBS) buffer pH 7.2 and 0.5% (w/v) Tween 20 (Fisher, PA). The blots were immunoblotted for 1 h with anti-PAL serum in a 1:1000 dilution at 25 °C. The membranes were washed 3 times 10 min each with PBS with 0.5% Tween 20. The secondary antibody was a horseradish peroxidase conjugated goat IgG (whole molecule) affinity isolated anti rabbit (Sigma, St. Louis) at 1:1000 dilution at 25 °C. After a similar washing procedure explained before, the signal was detected by chemiluminescence by using Renaissance reagents (DuPont NEN, Boston) on X-ray film (Fuji Super RX, Tokyo, Japan).

Example 7: Plant host cells and transgenic plants

Genomic libraries from *Lactuca sativa* are screened using probes from LsPAL1, and genomic clones are isolated which encode the gene for PAL. 5' regions associated with this gene are isolated and used in constructs to direct expression of proteins to region of wounding stress.

Plants are produced in which genes of interest are expressed at the site of, and in response to, physical, fungal, bacterial and pest-induced wounding of plant tissue.

Example 8: Antibody production

Fusion-PAL protein production, purification and separation were performed as previously described. The protein electrophoresis gels were stained with Coomassie brilliant blue and de-stained to visualize the bands. The gels were equilibrated several times in order to eliminate residues of de-staining solution. The selected bands were excised from the gel and divided in small pieces as described by Nonogaki et al. (1995), and extruded through a 25-gauge needle and stored at 4 °C until immunization of the rabbits. The extruded bands were injected subcutaneously in New Zealand rabbits at UC Davis Animal Resources Antibody Service. The first injection of the antibodies was performed with complete Feuds adjuvant and the subsequent were applied with incomplete Feuds adjuvant. The rabbit received six immunizations with the antigen varying the concentration between 0.6 to 0.2 mg/ml. The first 5 immunizations were performed with Factor Xa (New England Biolabs) cleaved fusion protein (1 ug Factor Xa per 50 ug fusion protein, at room temperature for 24 h). The last immunization was performed with fusion protein without protease treatment. The first 5 immunizations were injected at intervals of about 2 weeks. The last immunization was delayed until 4 weeks, and the exsanguination was performed by cardiac puncture 2 weeks later. The collected serum was aseptically filtered through 0.22 u low protein retention membranes and stored to previous utilization.

Example 9: Identification of PAL specific antibodies

The immunoprecipitation experiments was performed spectrophotometrical and by gas chromatograph mass spectrometry (GC-MS) analysis. For the spectrophotometric experiments, 50 g of lettuce 1-cm mid-rib tissue was stored for 24 h at 10 °C. The tissue was ground as previously described for the PAL activity assay. The supernatant resultant from centrifugation was mixed 1:2 (v/v) with cold acetone (-20 °C), and allowed to sit undisturbed for 1 h to precipitate soluble proteins. The mixture was centrifuge for 20 min at 5000 x g at 4 °C. The pellet was air-dried and dissolved in 5 ml 50 mM phosphate buffered saline (PBS) buffer pH 7.2. The methodology used to incubate acetone precipitated is described as follow: 150 uL of dissolved pellet in PBS was mixed with 5 uL of a serial dilution of anti-PAL serum. The serum cocktail (soluble proteins) was incubated for 24 h at 4 °C (modified from Walter, 1989). The mixture was centrifuged at maximum speed for 10 min in a bench top centrifuge at 4 °C. The supernatant (65 uL) was mixed with cold 50 mM borate buffer pH

8.5 to complete a volume of 1 ml. The resultant 1 ml was used to measure PAL activity, and the activity was expressed as nmol of cinnamic acid per ml produced in 1 h.

For GC-MS analyses, 2 g of lettuce mid rib (1 cm) were stored for 24 h at 10 °C. The tissue was ground as was described for the PAL assay. The supernatant resultant from centrifugation was mixed 1:2 (v/v) with cold acetone (-20 °C) for 1 h to precipitate soluble proteins. The mixture was centrifuge for 20 min at 5000 x g at 4 °C. The pellet was air-dried and dissolved in 1 ml 50 mM phosphate buffered saline (PBS) buffer pH 7.2. The methodology used to incubate acetone precipitated is described as follow: 100 uL of dissolved pellet in PBS was mixed with 10 uL of pre-immunization or anti-PAL serum. The cocktail serum (soluble proteins) was incubated for 1 h at 30 °C followed by 4 °C overnight (modified from Walter, 1989). The mixture was centrifuged at maximum speed for 10 min in a bench top centrifuge at 4 °C. The supernatant (40 uL) was mixed with cold 50 mM borate buffer pH 8.5 to complete a volume of 1 ml. The resultant 1 ml was used to measure PAL activity. After 1 h, saturated NaCl solution was added to product 1 M, and a drop of 1N HCl was added to reduce the pH ~2. The PAL activity mixture was spun in a top bench microcentrifuge at maximum speed. The supernatant was mixed with 10 uL of 0.1 ug ul^{-1} of syringaldehyde (Sigma, MO). Two ml of chloroform was added to the mixture and shaken for 2 min. The aqueous phase was removed and the chloroform phase evaporated with flow of N_2 until dryness. The dried material was dissolved in 100 uL of acetone. A 1.8 uL portion of the un-derivatized acetone solution was analyzed by GC-MS as describe by Fritz and Moore (1987) with modifications. The phenolic compounds were separated on a HP-5MS capillary column (30 m × 0.25 mm × 0.25 um) on a Hewlett Packard 5890 gas chromatograph coupled to an HP 5973 mass spectrometer operating in electron impact mode. The initial column oven temperature was set to 100 °C, with 5 °C min^{-1} increment to get a final temperature of 250 °C with 5 min hold at the maximal temperature. Peak area were determined using HP Chem Station software adapted for mass analysis. Spectra were recorded at 70 eV with the source at 200 °C. Authentic trans-cinnamic acid and syringaldehyde (Sigma, MO) were used as standard and internal standard respectively. Spectra of each were verified using the NIST spectral library. The linearity of area to mass relationship was confirmed with standards over a range from 0.001 ug to 1 ug.

The Ouchterlony double diffusion assay was performed as described by Bailey (1984) with the difference that PBS was used instead of barbitone buffer. A 5 uL portion of the

fusion PAL protein (approximately 0.6 mg ml^{-1}) was used as an antigen in the external wells and 5 uL of anti-PAL of serum was loaded in the center well

Example 10: ELISA specific for lettuce PAL protein

Measuring PAL protein levels in lettuce tissue by ELISA can be used as a means for identifying the propensity of lettuce tissue to brown, wherein a high level of PAL compared to control unwounded tissue or a tissue from a plant not susceptible to browning, would indicate that browning would occur within a few days. A relatively low level of PAL would indicate that a plant was less likely to brown within a specified time frame.

The anti-PAL antibody is coated and/or bound to a solid support structure (e.g. microtiter plates, plastic tubes or membrane). The particular support structures allow for a 'field test' to be developed that requires minimal equipment. The lettuce tissue to be tested is homogenized to a liquid state and then added to the solid surface that the anti-PAL antibodies are bound to, allowing the antibodies to bind the PAL protein in the homogenized lettuce tissue. After removing the homogenized lettuce tissue a secondary labeled anti-PAL antibody is added wherein the secondary antibody binds to the captured PAL protein from the lettuce tissue. A detectable signal is amplified by the addition of an appropriate enzyme (e.g. peroxidase or phosphatase) and an appropriate substrate added (e.g. ABTS or TMB). The amplified signal is measured using a spectrophometer as in the case of the microtiter plate and tubes or visually wherein standards are provided that allow for relative comparison. A format utilizing tubes and membranes, as appropriate, is used for a relative comparison.

Conclusions

The above examples demonstrate the isolation of two phenylalanine ammonia-lyase (PAL) sequences (polypeptide and nucleotide) from lettuce, wherein the sequences are induced by wounding. The PAL sequences were subsequently sub-cloned into an expression vector and determined to retain their enzymatic activity. The PAL amino acid sequences were also used as an immunogen wherein polyclonal anti-PAL antibodies were generated. These antibodies have specific utility as key components in an ELISA for the measurement of PAL protein levels in lettuce tissue.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claim.